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13. ABSTRACT (Maximum 200 Words)
Chromosomal rearrangements which result in localized increases of genetic material are frequent in breast cancer, and occur consistently in certain genomic regions. The resulting increase in expression of genes contained within these amplifications contributes to the malignant phenotype. Such amplified genes, such as Her2-Neu, provide targets for diagnosis and for the development of inhibitory drugs. The purpose of this study is to use novel genomic technologies to find new genes in breast cancer that are both highly amplified and are suitable targets by virtue of the fact that they are membrane-associated (receptors, membrane antigens, secretory proteins). The aims of the study are (1) To specify intervals of genomic amplification (amplicons) in primary breast cancer cell lines using genomic microarrays; (2) To prepare a database of membrane-associated genes, selected by differential hybridization of RNA prepared from fractionated microsomes; (3) To use the database to select membrane-associated genes that are located within amplicons, and measure their expression in the primary cell line using cDNA arrays, in order to select those that are upregulated. These genes will provide new insights and reagents for diagnosis and treatment of breast cancer.

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Table of Contents

INTRODUCTION

Chromosomal rearrangements which result in localized increases of genetic material are frequent in breast cancer, and lead to increased expression of genes within them. The resulting phenotypic changes in the cancer cell may provide it with a selective advantage, such as resistance to therapy (e.g. MDR-1 gene), increased growth potential (MYC gene), or unregulated growth due to autocrine stimulation (HER-2-Neu). The cloning of the HER-2Neu gene from an amplified genomic interval, and its subsequent use as a disease marker and treatment target, is one of the success stories in cancer research. Amplifications may be random mutational events, yet the consistent appearance of the same amplification in different specimens suggests that its consequences are of biological significance, indicating a gene of critical importance. It is therefore of considerable interest to find these highly amplified genes, with their potential as diagnostic markers and targets for therapeutic intervention.

Here we propose a strategy to find new targets from amplified chromosomal regions in breast cancer. Our research goal is to identify a subset of candidate genes that are both membrane-associated and contained in amplicons, and then test whether their expression is upregulated in breast cancer. To do this we will combine two novel approaches that utilize microarray technology. The first approach is to map regions of chromosomal amplification using genomic (DNA) arrays, applied to a panel of breast cancer cell lines created at our institution; this will provide our amplicon map. The second approach is to prepare a database of membrane-associated genes by preparing probes from fractionated ribosomes in a breast cancer cell line, and applying these to Affymetrix cDNA arrays; this will provide our breast cancer membrane gene database. The two data sets will then be compared to select genes which are likely to be amplified based on their genome sequence position, and their expression will be measured in the cell lines containing amplicons, to determine which are upregulated, confirming them as candidate. At the end of this study, we will have identified a set of genes, many of which are novel ESTs that have potential utility as clinical markers and treatment targets.

BODY

Progress on Task 1.

Task 1: To specify intervals of genomic amplification in 44 breast cancer cell lines or explants using genomic microarrays

a). to analyze 16 primary breast cancer cell lines and 28 xenografts with a 1-Mb resolution genomic microarray (Month 1-20)

b). to analyze the hybridization data for amplicon boundaries, minimal interval and amplicon peaks, in order to specify the genomic sequence positions of at least 25 recurrent breast cancer amplicons (Month 20-24)

Our colleague, Dr. R. Mehta, has begun the culture of the breast cancer cell lines, and DNA and RNA have been extracted from 8 samples so far. Half of the cases were established

from the primary cell line, and half from metastatic tissue. Note that these are all early passage cultures of breast cancer cells, and are expected to be "close" to the tumor in their genomic composition and gene expression, in contrast to commercially-available high-passage cell lines. Not all 16 cases were viable on thawing, but so far 8 are growing acceptably and the quality is excellent. The xenografts are disappointing because their viability is low, and there is a large admixture of murine stroma; thus, they will not be suitable for genomic analysis because the murine tissue will interfere.

We have performed a test hybridization of DNA to a Spectral Genomics 3-Mb array, using DNA extracted from the breast cancer cell line MDA231, and compared it to published results for CGH performed on the cell line (1). The results for two chromosomes are shown in figure 1 below. As can be seen in the image, the array easily detected the chromosome 6 amplification and the chromosome 9 deletion. Of note, the amplified region on chromosome 6 appears to resolve into two discrete regions (6q12-6p21; 6p21-6p23), which cannot easily be specified by CGH. We believe that using the higher resolution chips (1-Mb chip) would allow us even more accuracy in specifying the genomic interval than we can obtain on these 3-Mb chips. Our other conclusion is that the chips perform well, but there is considerable noise, and the study would benefit if all data were collected in duplicate. There has been delay in obtaining the 1-Mb chips. I contacted the manufacturer, who informed me that they are in production, and they will be ready by August 2003.

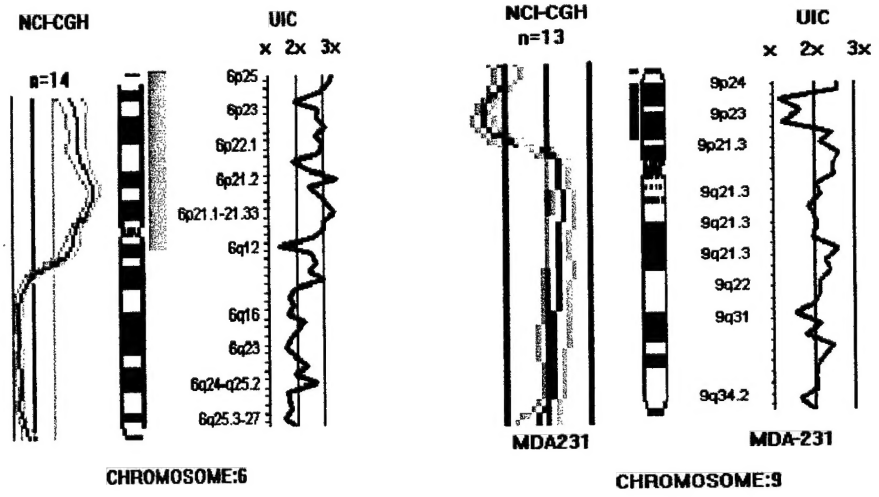

Figure 1. Test of Spectral Genomics 3-Mb array on breast cancer cell lines MDA231. The panels show results for two chromosomes, as indicated. Each panel compares the published results from Comparative Genomic Hybridization (CGH), on the left, to the results obtained with hybridization to a Spectral Genomics 3-Mb DNA array, performed at the UIC Genomics facility, on the right. The approximate region where the cell line deviates from normal is shown as bar next to the chromosome, with loss shown to the left and gain (amplification) to the right. The number of metaphases counted for CGH is indicated. The DNA hybridization graph reports the signal on the array, relative to control DNA, where 2x = normal diploid, 1x = loss of one chromosome, and 3x or higher = chromosome gain (amplification).

Progress on Task 2.

Task 2: To prepare a database containing at most 9,000 to 15,000 genes expressed in the MCF7 breast cancer cell line that are likely to be membrane-associated.

a). to extract membrane-bound and cytosolic RNA from the breast cancer cell line MCF7 and evaluate the quality of the separation by real-time PCR (Months 1-4)

b). to generate RNA from each fraction and validate its quality and its derivation from the membrane (Month 5-7)

c). to hybridize each RNA sample to the U95 set of 5 GeneChips and analyze the data to generate a membrane/cytosolic (m/c) ratio for every expressed gene, selecting those that are most likely to be membrane-associated (Month 8-12)

d).to prepare the surface-expressed gene database, including UniGene cluster number and sequence position on the genome map (Month 12)

Ribosome fractionation was performed essentially as described by Diehn (2). MCF7 cells were cultured to $5x10^8$ cells, and gently lysed (by Dounce homogenization) in a hypotonic solution so as to not disturb the ER association with the polysomes. Following nuclear precipitation, the remaining cell lysate is loaded on a discontinuous sucrose density gradient with ribonuclease inhibitors and spun in an ultracentrifuge for 5 hours at >25,000 x g to separate the membrane-bound polysomes (MBPs) from the free polysomes (FPs). MBPs, being less dense, rise to the top of the gradient, while the heavier FPs remain near the bottom of the gradient. Successive 1.5 ml fractions are collected from the sucrose gradient separately, and examined for RNA content by checking the absorbance at 260nm. Figure 2 shows that, we have successfully fractionated the RNA into separate populations; samples 2-15 are pooled as the FPs, while samples 20-26 are pooled as the MBPs.

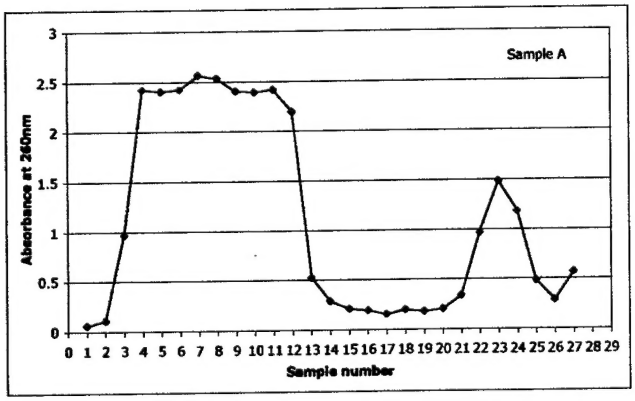

Figure 1. UV spectroscopy of fractions recovered from sucrose density gradient. Sample number corresponds to the order each sample was extracted from the bottom of the gradient. Samples 2-15 are pooled as the free polysomes (FP), while samples 20-26 are pooled as the membrane-bound polysome (MBP).

Fractions containing the membrane-bound and cytosolic polysomes were pooled. As a quality control measure to indicate that the two populations of RNA correspond to MBPs and FPs, real-time quantitative RT-PCR was performed using two primer pairs expected to amplify coding sequences specific for each population. Junctional adhesion molecule (JAM) is primarily cell surface associated, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a protein found free in the cytoplasm. Due to their different biological sequestering, JAM should be more associated with MBPs, while GAPDH should be more associated with FPs.

Total RNA was extracted using Trizol Reagent, and was purified using RNeasy columns. The quantity of total RNA was determined by A_{260} absorbance and agarose gel electrophoresis using standard techniques. For generation of first-strand cDNA, approximately 1 μg of RNA was reverse-transcribed using Superscript II Reverse Transcriptase (RT) Kit using oligo-dT priming. Real time PCR reactions were performed using DNAse-free cDNA templates generated above and SYBR Green PCR Core Reagents according to our usual laboratory protocols. Reactions were performed using an ABI Prism 7700 Sequence Detection System, and analyzed according to ABI guidelines. A cDNA template prepared from unfractionated MCF-7 RNA was used to generate a standard curve, and all test samples were normalized to 18sRNA as an internal control The sequence of the primers used in real time RT-PCR is as follows: endogenous control 18S ribosomal RNA (18SRNA): F:5'-GTAACCCGTTGAACCCCATT-3', R:5'-CCATCCAATCGGTAGTAGCG-3'with the expected size of 150 bp; JAM: F:5'-CCCTCTTGGCTTGATTTTGC-3', R:5'-TGACCTTGACTGATGGCTTC-3' with the expected size of 115 bp. The GAPDH primers were obtained from Applied Biosystems (Foster City, CA)

Quantitative measurements of JAM and GAPDH for each of the two fractions are shown in Table 1. When the ratios are compared, it is clear that GAPDH is enriched compared to JAM in the CP (GAPDH/JAM ratio 119), while the inverse is true in the MBP (GAPDH/JAM ratio 0.199). Thus we conclude that the fractionation method successfully separated membrane-associated RNA from cytosolic RNA, as predicted.

	JAM	GAPDH	JAM/GAPDH	GAPDH/JAM
Cytoplasn Fractior	5.4633	652.57	0.008	119.446
Membrane Fr	2.1133	0.42	5.032	0.199

Table 1. Realtime RT-PCR measurements of JAM and GAPDH in RNA prepared from the CP and MBP, expressed relative to 18S RNA. Ratios of measurement are shown in the right two columns.

These two pools will be hybridized to Affymetrix arrays (U133A chips). The UIC Genomic Facility is experienced in Affymetrix hybridizations, and our lab has hybridized and analyzed over 20 chips to date. The hybridization and database preparation are expected to be completed within the next 2 months.

Progress on Task 3:

Task 3. To select genes within amplicons and measure their expression level, to identify those that are upregulated

a). to select membrane-associated genes that are within amplicons, comparing the dataset in Aim 2 with the sequence location of amplicons from Aim 1 and the literature (Month 25)

b). to prepare the cDNA array (Month 25-27)

c). to perform and expression analysis of 44 breast cancer cell lines and xenografts on the cDNA arrays (Month 28 -33)

d). to analyze the expression data collected here, and survey available databases, to identify highly expressed genes (Month 33-36)

These tasks will begin in Year 3. There is no progress to report.

KEY RESEARCH ACCOMPLISHMENTS

1. Prepared DNA and RNA from 8 primary breast cancer explant cultures
2. Tested Spectral Genomics arrays and demonstrated their utility and limitations.
3. Fractionated microsomes from the breast cancer cell line using sucrose-gradient ultracentrifugation and prepared from them.
4. Verified by realtime RT-PCR that the RNA has been appropriately separated into membrane and cytosolic fractions.

REPORTABLE OUTCOMES

None.

CONCLUSIONS

Task 1: We have kept to the proposed timeline for Aim 1.We have prepared DNA from 8 cell lines and tested our chips, but have not completed the full hybridization study. Based on the experience of our collaborator, Dr. R. Mehta, we are assured of the quality of the lines that are viable, but some have not grown well after thawing. Also, we find that the xenografts will not be suitable because of the high murine tissue content. Based on this, we now anticipate having 8-12 lines available for array studies. Nonetheless, these are valuable reagents since they are early passage and they are expected to closely resemble the tumor, unlike other high-passage cell lines that are in widespread use. Furthermore, they are well-characterized clinically, and represent both breast primaries and metastatic tissue. The other accomplishment is that our chips were tested, and we demonstrated that we can adequately prepare the sample and collect the data, but we now feel that it will be necessary to do each sample in duplicate. There may be some delay in completing this aim, depending on manufacturer's ability to supply the chips in a timely fashion.

However, we expect that collection of data will go quickly when this task is begun. If the 1-Mb chip is not produced, we will use the 3-Mb chip, which is available and will be suitable though resolution is lower.

Our revised Task 1a

(a) to analyze 8 – 12 primary breast cancer cell lines, in duplicate on DNA array chips (Month 1-20)

Task 2: We are slightly behind in the preparation of the database, but have successfully completed 2a and 2b. We have demonstrated our ability to separate the RNA into cytosolic and membrane fractions and verified the quality. All that remains is to hybridize the Affymetrix chip, collect and analyze the data, and assemble the database; our genomic facility is very experienced in this methodology. No modification of Task 2 is anticipated, and we expect to complete it by Month 18.

Task 3: Begins in Year 3. No modification is proposed.

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1. Forozan, F., et al., *Comparative genomic hybridization analysis of 38 breast cancer cell lines: a basis for interpreting complementary DNA microarray data.* Cancer Res, 2000. **60**(16): p. 4519-25.

2. Diehn, M., et al., *Large-scale identification of secreted and membrane-associated gene products using DNA microarrays.* Nat Genet, 2000. **25**(1): p. 58-62